

Research report

Chronic tooth pulp inflammation causes transient and persistent expression of Fos in dynorphin-rich regions of rat brainstem

Margaret R. Byers^{a,b,*}, Eric H. Chudler^a, Michael J. Iadarola^b^a Department of Anesthesiology, University of Washington, Seattle, WA 98195-6540, USA^b Neuronal Gene Expression Unit, Pain and Neurosensory Mechanisms Branch, NIDCR, NIH, Bethesda, MD 20892, USA

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Abstract

We have analyzed central Fos immunoreactivity (Fos-IR) brainstems of adult rats after three clinically relevant dental injuries: filled dentin (DF) cavities that cause mild pulp injury and heal within 1–2 weeks; open pulp exposures (PX) that cause gradual pulp loss and subsequent periodontal lesions; and filled pulp exposures (PXF). By 1 week after DF cavities, no Fos-IR remained except for sites such as lateral–ventral periolivary nucleus (LVPO) that had Fos-IR in all rats including controls. PX injury induced (1) a delayed transient expression of Fos at 1–2 weeks at three loci (ipsilateral neurons in dorsomedial nucleus oralis, paratrigeminal nucleus, and trigeminal tract), (2) persistent ipsilateral Fos for at least 4 weeks after injury in dynorphin (Dyn)-rich regions (rostral lateral solitary nucleus, peribex dorsal nucleus caudalis), and (3) late Fos-IR at 2–4 weeks (bilateral superficial cervical dorsal horn, contralateral dorsal nucleus caudalis, contralateral rostral lateral solitary nucleus). Rats with PXF injury were examined at 2 weeks, and they had greater numbers and more extensive rostro-caudal distribution of Fos neurons than the PX group. One week after PX injury, Fos-IR neurons were found in regions with strong Dyn-IR central fibers. Co-expression of Dyn and Fos was found in some unusually large neurons of the ipsilateral rostral lateral solitary nucleus, trigeminal tract, and dorsal nucleus caudalis. Immunocytochemistry for the p75 low affinity neurotrophin receptor (p75NTR) or for calcitonin gene-related peptide (CGRP) showed no consistent change in trigeminal central endings in any Fos-reactive brainstem areas, despite the extensive structural and cytochemical reorganization of the peripheral endings of the dental neurons. The Fos responses of central neurons to tooth injury have some unusual temporal and spatial patterns in adult rats compared to other trigeminal injury models. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Dental injury; Pain; Lateral solitary nucleus; Nucleus oralis; Nucleus caudalis; Trigeminal tract interstitial cell

1. Introduction

Dental injury models are useful for correlating the degree of peripheral injury with the induced changes in trigeminal ganglion and central nervous system, because the depth and location of dentin and pulp injury can be controlled to cause specific amounts and duration of local injury, inflammation, systemic defense, wound healing, and neural reactions [2,12,14,20,46,71–74]. For example, dentin cavity preparation causes a transient pulpal and neural response for a few days, while pulp exposure causes persistent, progressive inflammation that gradually de-

stroys the pulp and spreads into periodontal tissues with persistent neural sprouting and neuropeptide changes in the surviving tissues. Both types of injury induce upregulation of pulpal nerve growth factor, local inflammation, and altered trigeminal expression of neurotrophins and their receptors [16,78], while only pulp exposure induces expression of glial fibrillary acidic protein in satellite cells of trigeminal ganglion [68] and increased expression of the α_1 subunit of calcium channels in trigeminal neurons [77]. In the present study, we extend this type of analysis to the brainstem to determine whether central neurons have persistent Fos expression after these types of tooth injury.

When neurons undergo changes in phenotype, the phosphoprotein transcription factors Fos and Jun accumulate in the nuclei where they can be detected within 1–2 h by biochemical or immunocytochemical methods [24, 37,56,65]. Numerous studies have shown that stimulation

* Corresponding author. Department of Anesthesiology, Box 356540, University of Washington, Seattle, WA 98195-6540, USA. Fax: +1-206-685-3079; e-mail: byersm@u.washington.edu

of particular afferent pathways, including pain pathways [37], causes specific somatotopic patterns of Fos immunoreactivity (Fos-IR) in CNS neurons undergoing plastic changes (e.g., see Refs. [4,5,29,81]). For example, the Fos-IR patterns induced by cutaneous noxious stimulation [8,9,70,83] differ from those of articular or visceral injury sites [6,34,50,52,75,83] vascular inflammation [69] or intraoral injury [71]. Hunt et al. [37,57,79] were the first to demonstrate that different sites and intensities of noxious stimulation cause specific patterns of Fos-IR in superficial neurons of the dorsal horn. Many other studies have demonstrated especially strong Fos-IR reactions to chronic inflammation or peripheral nerve constriction, involvement of capsaicin-sensitive nerve fibers in generating a Fos-IR response, importance of excitatory (NMDA) channels for activating Fos in the responding CNS neurons, prominent involvement of dynorphin (Dyn) and enkephalin interneurons, and correlations with hyperalgesia, morphine inhibition, adrenergic activation and central opioid mechanisms [1,2,10,20–22,26–28,31,32,36,38–40,43,58,61,82]. The Fos-IR response in dorsal horn is enhanced by larger injury [42], and can be reduced by central activity induced by stress [33] or by descending modulation [62]. There are important differences in the time course of a variety of DNA-binding proteins in the Fos, Jun and Krox families (c-Fos, Fos-B, Jun-B, Jun-D, c-Jun, KROX-24) after peripheral nerve stimulation or tissue inflammation [35,49]. These differences have been correlated with specific stages of inflammatory disease such as initiation, flare-up or chronic progression [49]. Thus, mapping of DNA-binding proteins and especially Fos has become a powerful tool for understanding the linkages between the CNS, peripheral nociceptive nerve fibers and stages of peripheral inflammation and healing (for reviews, see Refs. [33,57]).

Several studies of acute Fos responses of trigeminal subnuclei to tooth injury have shown that (1) the Fos response in the periobex regions of nucleus caudalis is intense by 2–4 h after tooth extraction or pulp exposure [76], (2) the number of Fos responding neurons depends on stimulus, timing and intensity [18,22,23,42,76], (3) stimulation of C-fibers causes different Fos patterns than specific stimulation of A- δ dental fibers [19,59], (4) the expression of Fos in trigeminal nuclei is independent of altered motor reflex responses [42], (5) the responses are inhibited by opioids [2,20], (6) the main trigeminal nucleus and subnuclei interpolaris and oralis usually have no Fos response (but see Sugimoto et al. [72]), and (7) there is

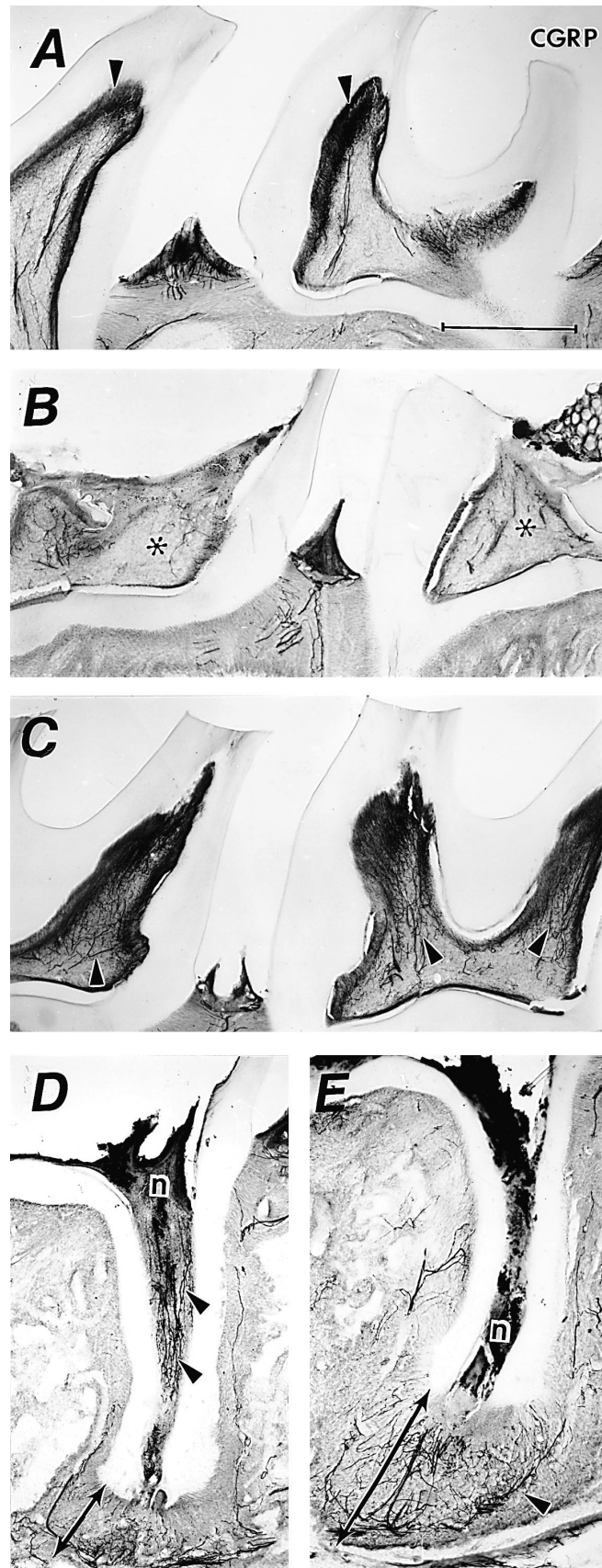
Table 1
Animal groups

Treatment	Post-injury time	<i>n</i>
Intact	–	8
DF/sham	1–2 weeks	6
PX/open	Acute (5–7 h)	14
PX/open	Chronic 1 week	14
	Chronic 2 weeks	7
	Chronic 4 weeks	7
	Chronic 2 weeks	3
PXF/filled	Chronic 2 weeks	3

greater rostro-caudal response when more teeth are involved [42]. In a preliminary report, we demonstrated that 2–4 weeks after pulp exposure injuries to rat molars there was persistent bilateral Fos in the superficial layers of the dorsal periobex region of nucleus caudalis and in the rostral lateral solitary nucleus, and those patterns did not colocalize with degenerating afferents that were shown by silver techniques [15].

In the present paper, we have analyzed Fos responses from the cervical cord throughout the medulla using an antibody that recognizes Fos and Fos-related antigens [26,41,81], designated ‘Fos’ in this paper. Central neuronal responses to tooth injury were also analyzed in serial sections for Dyn, calcitonin gene-related peptide (CGRP) and low affinity p75 neurotrophin receptor (p75NTR) IR to determine whether some of the Fos-IR neurons co-expressed Dyn and whether there were alterations in primary afferent arborization or cytochemistry near the Fos-IR neurons. Earlier mapping of dental afferent terminals in brainstem found a wide distribution extending from the cervical segments to the main sensory nucleus for the first rat molar [51], as would be expected from the numerous electrophysiological studies of responses in all subnuclei of the trigeminal system to tooth stimulation [25,48,66] and by the widespread [14 C]-2-deoxyglucose labeling after rat incisor stimulation [67]. However, the various Fos responses to tooth injury were mostly limited to narrow subregions within that dental nerve arborization. The Fos patterns shifted over time, as the peripheral lesion moved beyond the crown pulp and into periodontium, and there were interesting features of the responses related to severity of injury, size of reactive neurons, and the lack of structural plasticity in the central terminals of the dental afferent neurons despite their extensive peripheral alteration near the tooth injury site.

Fig. 1. CGRP-IR nerve responses to injury of adult rat molars. (A) Normal distribution and immunoreactive intensity of CGRP nerve fibers is seen here in intact first and second molars of a control jaw. Arrowheads: CGRP-IR nerve ending region in dentin and peripheral pulp. (B) At 5 h after PX injury, the CGRP contents in surviving pulp (*) and nerve fibers was greatly depleted. (C) At 1 week after DF injury, the neural CGRP intensity and branching extent was enhanced for crown pulp nerve fibers (arrowheads). (D) At 1 week after PX injury, all the crown pulp has been lost to necrosis (n), but CGRP-IR nerve fibers were found (arrowheads) in surviving root pulp, and in periodontal regions outside the root apex where there was initial alveolar bone loss (double-headed arrow). (E) At 4 weeks after the PX injury, the pulp was completely necrotic (n), an enlarged area of bone loss (double headed-arrow) was present, and nerve sprouting (arrowhead) is extensive outside the affected root. Scale bar: 0.5 mm (in A; A–E at same magnification).



2. Materials and methods

2.1. Animals

These studies were approved by the University of Washington Animal Care and Use Committee and follow the animal experimentation guidelines of the International Association for the Study of Pain and the Society for Neuroscience. Anesthetized adult Sprague–Dawley rats ($n = 57$ male and 2 female) received a specific injury to the right maxillary first and second molars, described below, that consisted of filled cavities drilled partially into the coronal dentin (DF), open pulp exposures (PX), or filled pulp exposures (PXF), or they were uninjured controls (Table 1). For the PX group, post-operative times varied

from 5–7 h to 4 weeks. The DF rats were examined at 1 and 2 weeks, and the PXF group at 2 weeks. The Fos-IR responses in the DF group did not persist, and so those animals also served as sham controls for the PX and PXF groups, since they had similar anesthesia and jaw opening for tooth surgery. The group of PXF rats ($n = 3$) was added to this study because they had a reduced rate of recovery of post-operative weight gain (5–7 days for recovery compared to 2–5 days for PX group and 1–2 days for DF group). We wanted to know if their prolonged reduced feeding period was related to subsequent increased central Fos-IR neurons. The rats were housed with freely available food and water, ambient room temperature, and 12 h light/dark cycle.

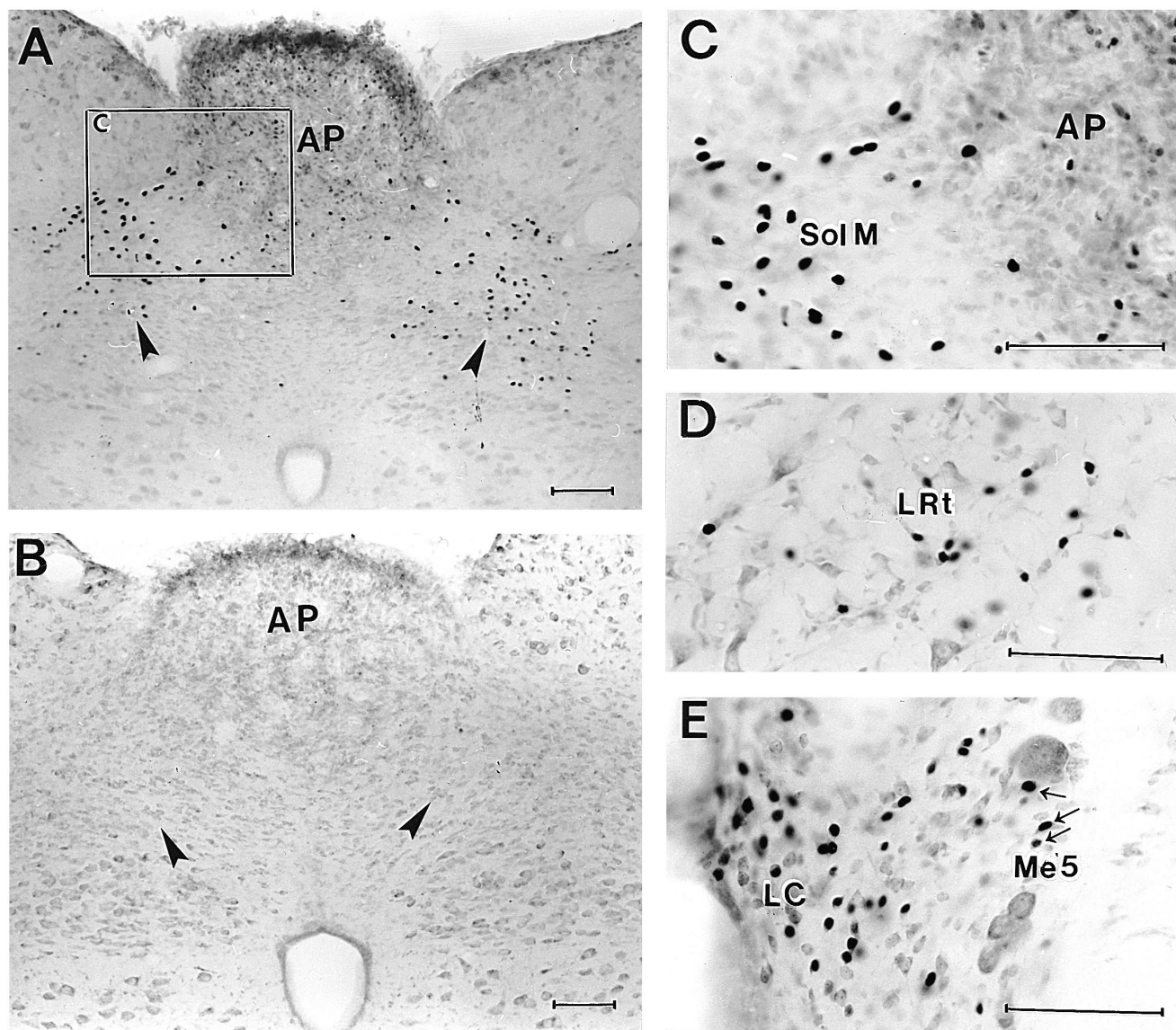


Fig. 2. Bilateral acute response. (A) Many neurons in bilateral medial solitary nuclei (arrowheads) had Fos-IR at 5–7 h after tooth injury. The Fos-positive neurons in AP were smaller than those of SolM. (B) There was a lack of Fos-positive neurons in SolM of control rats and in those injured 2–4 weeks (4 week injury shown here). (C) High magnification of Fos-positive neurons in SolM and AP. (D) Neurons in LRt. (E) Some rats had Fos-IR neurons in bilateral locus coeruleus (LC), and small Fos-IR cells (arrows) near the unreactive large neurons of the mesencephalic trigeminal nucleus (Me5). Scale bars: 0.1 mm.

2.2. Dental injury

Surgical procedures were similar to those described earlier [16,45,46,68] and reviewed recently [12]. Specifically, each rat was anesthetized with i.p. Equithesin (0.97% sodium pentobarbital and 4.25% chloral hydrate, at 0.35 ml/100 g b.wt) and was placed on its back on a special board designed to hold the mouth open with a spring-mounted plastic ring fitting over the mandibular incisors and a rubber band holding the maxillary incisors. A small retractor held the cheeks apart and the tongue was placed gently to the left side with a cotton swab. An extra 0.1–0.3 ml Equithesin was given to some rats as needed. The injury groups (Table 1) are as follows: (1) *DF*: This group had half of the occlusal dentin overlying the right maxillary first and second molar pulps removed by gentle drilling with a sterile #2 round bur and then etched and filled with resin (Concise, 3 M, St. Paul). These rats survived 1 or 2 weeks. They served as our sham controls because this procedure did not cause central Fos response after the first few days. (2) *PX*: The coronal pulps of the first and second right maxillary molar teeth were exposed by removing overlying occlusal dentin using an air-cooled, high-speed hand piece and a new #2 round bur for each rat. Only 0.5–1 min of gentle tapping action was needed to expose the pulps, and no other oral tissue injury occurred. The two teeth remained open for 5–7 h, or 1, 2, or 4 weeks. (3) *PXF*: The injury was the same as for group two, but then the exposures were treated by placing calcium hydroxide crystals on the pulp as is typical for clinical endodontics, and then filled with resin (Cavit, from ESPE, Seefeld, Germany). (4) *Intact Group*: These rats, of which two were anesthesia controls and six untreated, were age-matched and housed together with each experimental group. Softened food pellets were provided during the first post-operative day. All operated rats were eating and drinking within the first hour after recovery from anesthetic, followed by a period of reduced feeding that usually lasted 1–2 days for anesthesia shams or DF injury and 2–5 days for PX injury [12]. The PXF group in the present study did not resume normal weight gain until 5–7 days after surgery.

2.3. Tissue fixation and preparation

Pilot studies at 1 week showed that there was transient Fos-IR if the rats were handled during the last 24 h. Thus, the rats in 1–4-week survival groups were not weighed or disturbed during the last day until receiving i.p. Equithesin anesthetic 15 min prior to perfusion fixation. Each anesthetized rat was perfused through the heart for 5–10 min with 250–300 ml of Zamboni fixative (4% paraformaldehyde, 0.2% picric acid, 0.1 M phosphate buffer, pH 7.4) using 5 ft of gravity pressure and no saline pre-wash. The rat was then cooled on ice for 30–60 min for additional tissue fixation prior to dissection. The jaws and the brainstem from the level of the inferior colliculus to the first

two cervical segments were removed and post-fixed another 4–12 h. The jaws were then decalcified in daily changes of formic acid solution (4 N formic acid in 0.5 M sodium formate, pH 2.5) for 1–2 weeks. We had learned from studies of developing dental innervation that there was no detectable loss of Fos-IR during several weeks in the formic acid solution [11]. We, therefore, routinely kept the tissue in cold formic acid solution for 1–2 weeks, while accumulating a set of 3–5 brainstems at different post-operative times, an intact (negative) control, and an acute injury (positive) control to process together for each immunocytochemical experiment. Two days prior to sec-

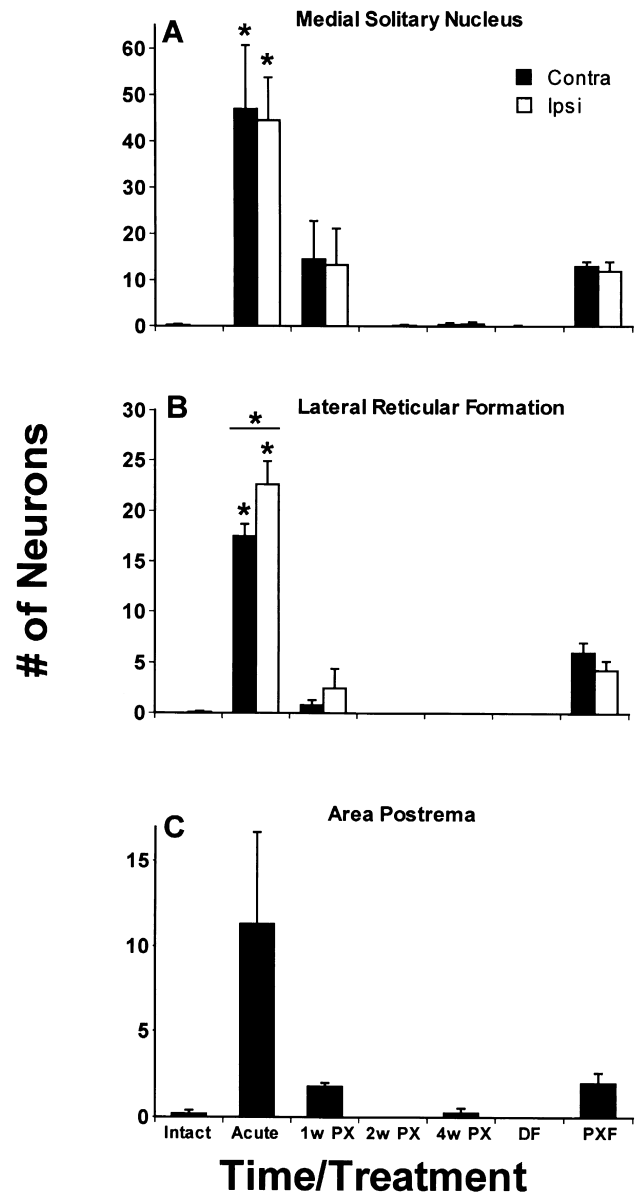


Fig. 3. Acute Fos-IR (mean number per section \pm S.E.M.). (A) The SolM response was bilateral in the acute group, with significant differences from other postoperative times (* $p < 0.05$). (B) There was a bilateral response ($p < 0.05$) in neurons of LRt, that was significantly greater for ipsilateral neurons (*, bar). (C) The acute trend in neurons of AP did not reach significance ($p = 0.065$).

tioning, the tissues were rinsed in 0.1 M phosphate buffer pH 7.4 and equilibrated in 30% sucrose. Transverse serial sections (50 μ m) were cut on a freezing microtome from the cervical cord all the way to the inferior colliculus. All sections were collected in phosphate-buffered saline as four sets (each set containing every fourth section per 200 μ m).

2.4. Immunocytochemistry

2.4.1. Jaw tissue

Longitudinal sections containing midline pulp of all three molar teeth per maxilla were selected from each rat. Nerve distribution patterns were detected by immunocytochemistry using a polyclonal antibody against CGRP (Cambridge Research Biochemical/Genosys) diluted 1:7500 and detected by the avidin–biotin–DAB procedure (Vector, Burlingame, CA), as described previously [46] (Fig. 1).

2.4.2. Brainstem

Initially, one of the four sets of sections was processed for Fos-IR. If the initial reaction had strong Fos-IR signal

at constitutive expression sites in all rats and at medial solitary nucleus (SolM) and lateral reticular formation (LRt) in acutely injured rats, then the other sets were processed for CGRP (1:7500), p75NTR (monoclonal IgG192 at 1:500) [13], or Dyn (1:4000). Some of the sections from 1-week PX rats were double reacted (Dyn/Fos or CGRP/Fos). If the Fos signal was weak, then that reaction was repeated at a higher concentration of primary antibody with an extra series of sections. The primary antibody for Fos-IR was polyclonal, made in rabbit against the highly conserved “M” peptide region (amino acids 129–153) that is common to c-Fos, Fos b and Fos related antigens Fra-1 and Fra-2 [26,41,81]. Several antibody preparations were made from serum from two rabbits, and each was tested in a dilution series to determine optimal antibody concentration. After blocking endogenous peroxidase by incubation in 3% H₂O₂ plus 90% methanol for 30 min, the sections were incubated free-floating in 1:2500–1:10,000 dilutions of primary antibody at 4°C for 2 days. Bound primary antibody was detected using biotylated goat anti-rabbit IgG second antibody (Vector), an avidin–biotin–peroxidase complex (Vector),

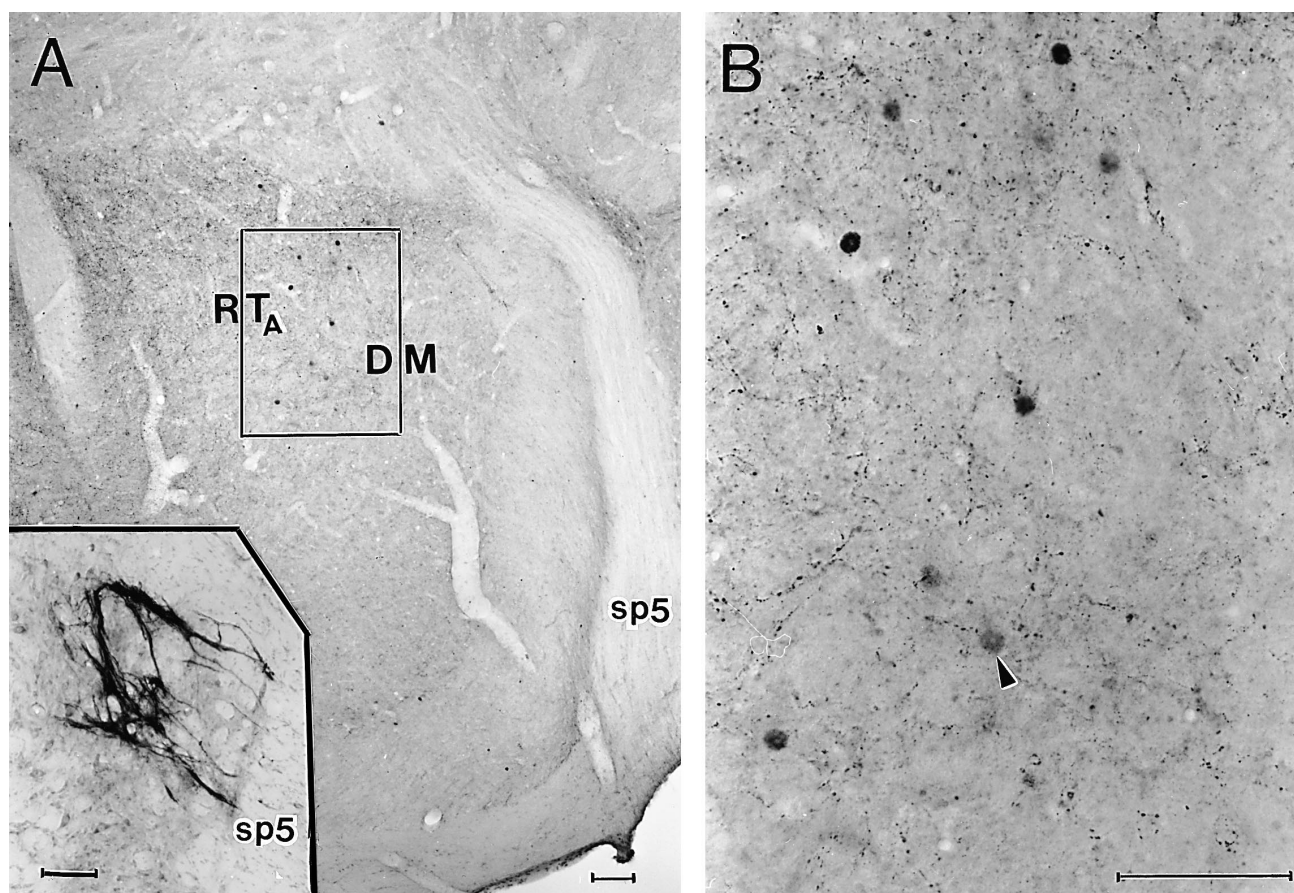


Fig. 4. Delayed, transient response in ipsilateral nucleus oralis. (A) This section was immunoreacted for both Fos and Dyn. Large neurons scattered within the dorsomedial (DM) region of nucleus oralis and in the adjacent reticular nucleus-_A (RT-_A) have Fos-IR nuclei, but not Dyn-positive cytoplasm. (B) Some of the reactive oralis neurons had pale nuclei (arrowhead) rather than the usual intense immunoreactivity as seen in the higher magnification of the region outlined in (A). Numerous beaded Dyn-IR fibers end in the region near the large Fos-IR neurons. sp5: Trigeminal tract. Inset: p75NTR-IR afferent fibers terminate in the RT-_A/DM region of nucleus oralis. Scales: 0.1 mm.

and nickel-enhanced diaminobenzidine (DAB, Sigma) with 0.0125% hydrogen peroxide. In all cases, we monitored the Ni-DAB in SolM neurons at $40\times$ magnification as it developed in sections from the acute PX positive control sample per immuno batch. The reaction was stopped when those SolM neurons were fully reacted Fos-IR (Fig. 2A). Specificity of the antibody was confirmed using pre-adsorption with Fos antigen as in Ref. [81] or by omission of the primary antibody (data not shown). For each set of immunoreactions, sections from 2–4 rats with chronic PX or DF injury were reacted together along with a negative control (uninjured intact group) and positive control (acute PX). The immunoreacted sections were mounted on gelatin-coated slides, dried, lightly counterstained with 0.005% basic methylene blue/Azure-II, dehydrated, and covered. The subsequent study of three PXF rats also included positive and negative controls.

2.4.3. Double-labeling

Double immunocytochemistry was performed on sections from two intact, one acute PX, one 7-day DF, and five 7-day PX rats. We first performed the Fos immunoreaction using Ni-DAB (black reaction), as described above. Half of those Fos-reacted sections were then re-incubated in primary anti-Dyn (1:4000) at 4°C for 2 more days and the other half were reacted for CGRP (1:7500). Both reactions for the second antigen were detected by standard DAB (orange/brown color). The antibodies for Fos, Dyn, and CGRP were all polyclonal made in rabbit. However, the Fos/Dyn or Fos/CGRP were clearly distinguished from each other because of the different colors and cellular compartments, and by the intensity differences from control sections incubated in non-immune serum.

2.5. Analyses and statistics

The number of Fos-positive neurons was analyzed blind using coded slides. First, we estimated Fos numbers for one set of 50 μm semi-serial sections (one per 200 μm) through the brainstems of all 59 rats at a magnification of $63\times$. Because of the low magnification and basophilic counterstain, the weakest Fos-IR cells were not detectable, and the numbers represent only medium and high intensity staining. The two sections showing the middle of each key nucleus were identified, and all Fos-IR neurons at those sites were re-counted for the 30 rats with the strongest immunocytochemical staining (intact controls, $n = 5$; acute 5–7 h PX, $n = 6$; 1 week PX, $n = 6$; 2 weeks PX, $n = 3$; 4 weeks PX, $n = 4$; DF at 1–2 weeks, $n = 3$; PXF, $n = 3$) using a Zeiss microscope at $63\times$ and camera lucida mapping. For cervical dorsal horn, there was greater variability and all Fos-IR neurons in five 50 μm sections (one section per 200 μm) were averaged (over 1 mm distance). For interstitial cells, sections from the entire medullary trigeminal tract (15 successive semi-serial 50 μm sections, 1:4 series over 3 mm distance) were analyzed per rat. Data

for each rat were expressed as average number of Fos-IR neurons per section and for each group as mean \pm S.E.M. The Fos-IR patterns in the other 29 rats were similar to those of the quantified group. Key representative cell groups were then photographed using a Nikon FXA microscope.

Sizes of the largest Fos-IR nuclei were determined using digital images at $1250\times$ (NIH-Image 1.62 software) for the 1-week PX group by outlining each nucleus and obtaining a cross-sectional area. The analysis included the

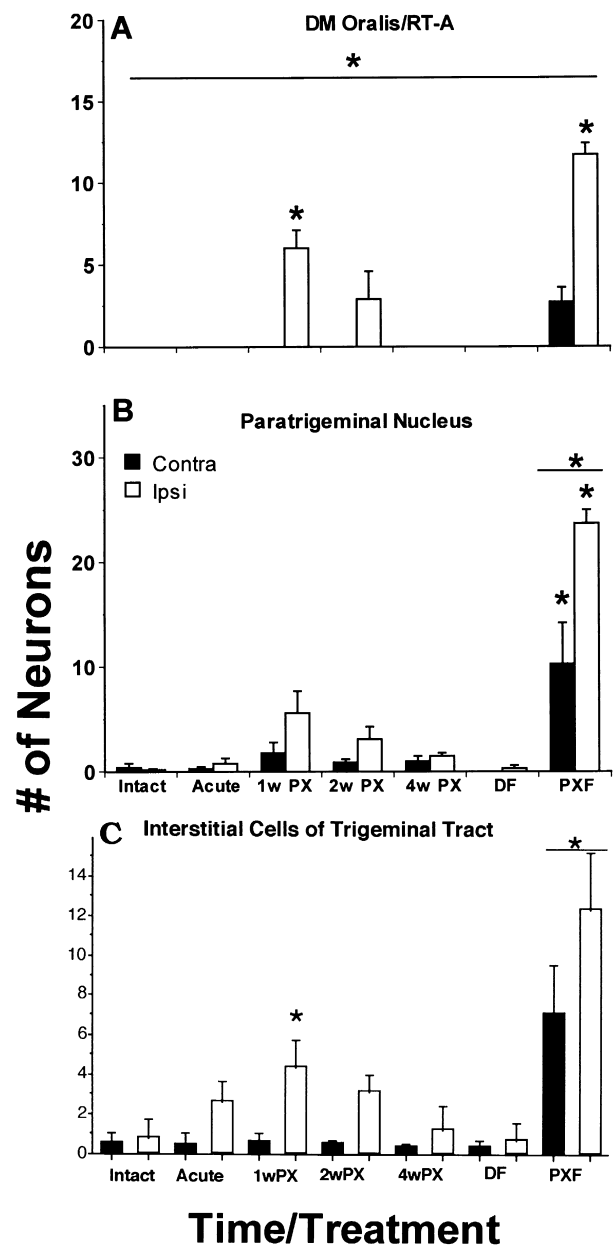


Fig. 5. Delayed transient response (mean number per section \pm S.E.M.) (* $p < 0.05$). The responses in the ipsilateral DM oralis/RT-A and interstitial cells of trigeminal tract were significantly different for the 1-week PX and 2-week PXF groups compared to the other groups. The paratrigeminal Fos-IR neurons were located along the edge of that nucleus and were only significant for the PXF group.

10 largest neurons per section for ipsilateral and contralateral nucleus oralis, rostral lateral solitary nucleus, periobex

nucleus caudalis at dorsal and ventral levels, and interstitial cells of the trigeminal tract. There was not a problem

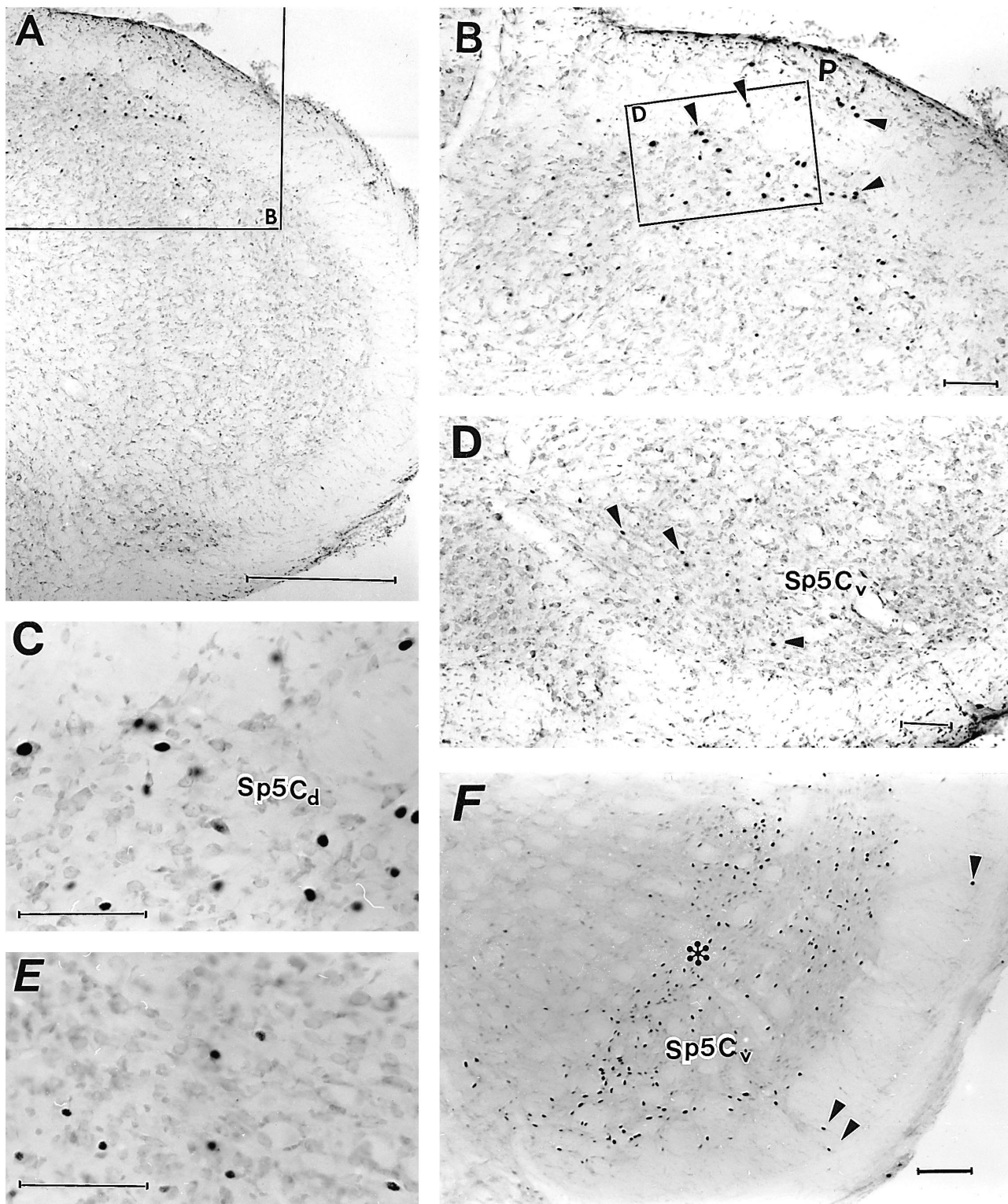


Fig. 6. Persistent Fos in the periobex region. (A–C) Fos-IR neurons are shown in ipsilateral periobex transition zone that includes dorsal nucleus caudalis (Sp5Cd, 2-week PX group). The paratrigeminal nucleus (P) was mostly unlabeled except for cells bordering the trigeminal tract (arrowheads, B). (D–E) Fos-IR neurons were small and sparse in the ventral nucleus caudalis of 2-week PX rats (Sp5 Cv). (F) Many more Fos-IR neurons occurred in nucleus caudalis of PXF group at 2 weeks than in PX group and included the ventral region and transition zone (*), as well as interstitial cells in trigeminal tract (arrowheads). Scale bars: 0.1 mm.

with cut profiles of the nuclei, because the sections were 50 μm thick and the measured nuclei were fully visible within the section, thus, allowing tracing of their widest perimeter.

Two-way analysis of variance (ANOVA) was used to determine the significance of the differences between numbers of Fos-IR neurons for different injury groups and times. Following a significant f -value, post-hoc paired comparisons between groups were performed using the Kruskal–Wallis test (Sigma Stat, software 2.03; and p -significance level was set at $p < 0.05$). The same significance testing was performed for the comparisons of post-operative weight gain for different injury groups and for the comparison of Fos-IR nuclear size at 1 week.

3. Results

3.1. Rat molar injury

After acute PX injury (5–7 h), the CGRP-IR of the dental nerves in the surviving pulp had not recovered from its injury-induced neuropeptide release (Fig. 1A–B) and nerve fibers with CGRP were rare. By 1 week after DF injury (Fig. 1C), the pulp was intact and the sensory nerve

CGRP-IR in the crown was greater than normal, as expected [73].

PX injury caused a gradual loss of pulp tissue over several weeks, with nerve fibers continuing to sprout in the tooth if there was surviving pulp and then shifting to sprouting reactions outside the tooth once most of the pulp was gone (Fig. 1D–E). The chronic PX injury is a completely different challenge to the animal than the easily repairable DF injuries, because it causes a persistent condition of progressive infection, gradual loss of pulp, continuing acute inflammation and sensory nerve sprouting reactions in the surviving tissue, and lesions in periodontal tissue starting by 5 days. The rate of pulp loss for the 2-week PX group was similar to the 2-week PXF group (not shown).

3.2. Constitutive expression of Fos in adult rat brainstem

In all control and injury groups, there was prominent bilateral expression of Fos by neurons in three areas: inferior colliculus, the A5 cluster of adrenergic neurons, and lateral–ventral periolivary nucleus (LVPO). The mean number of Fos-IR neurons in LVPO varied from 24–55 per section for all rats (data not shown). The reasons for these patterns of constitutive Fos expression are not known,

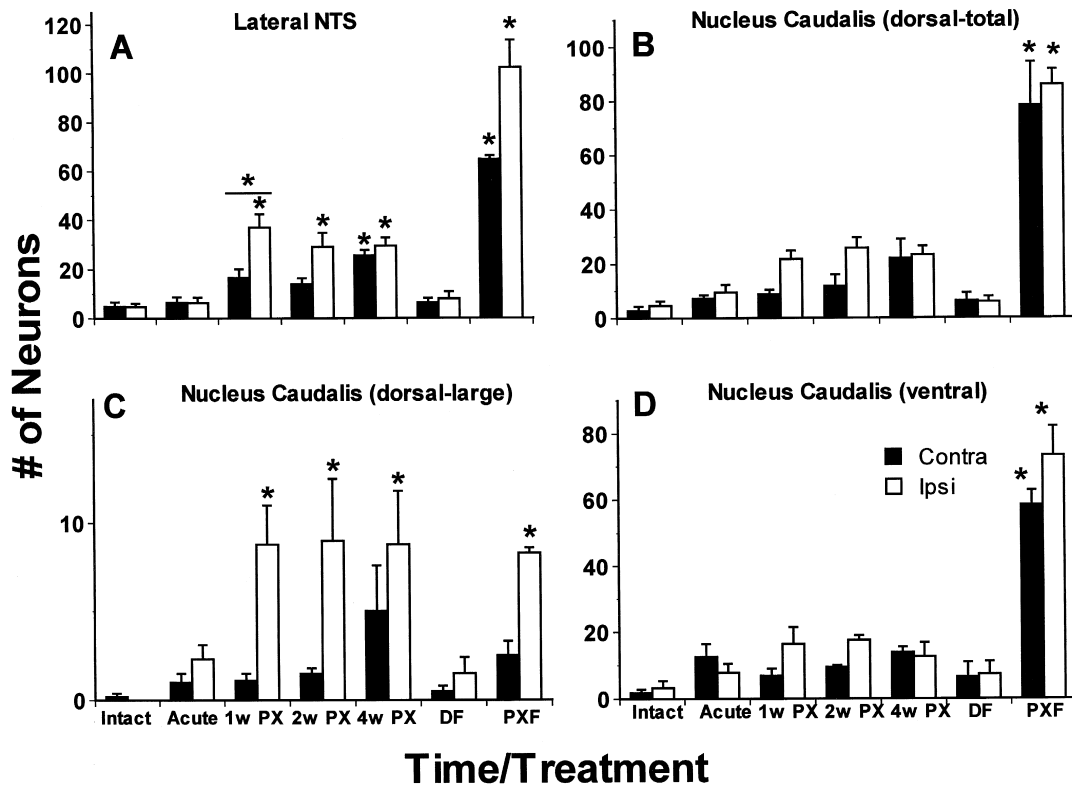


Fig. 7. Persistent Fos-IR responses to PX injury (mean number per section \pm S.E.M.) (* $p < 0.05$). (A) Fos-IR neurons were increased ipsilaterally from 1–4 weeks and contralaterally at 4 weeks for rostral lateral solitary nucleus. (B) The trend for increased Fos-IR neurons in ipsilateral nucleus caudalis was only significant for the PXF group. (C) The increase in neurons with large Fos-IR nuclei ($> 50 \mu\text{m}^2$ cross-sectional area) was significant for the ipsilateral groups. (D) The trends for ventral nucleus caudalis were only significant for the PXF group.

but they were useful positive controls for the immunocytochemical technique within each rat. The PXF group had a trend for greater LVPO response than DF or PX groups that was not significant.

3.3. Acute Fos patterns

Neurons in bilateral SolM and LRt had significant ($p < 0.05$) Fos-IR responses in the acute PX group, but not at later time periods and not in DF or PXF rats (Figs. 2 and 3). In area postrema (AP), dorsal parabrachial nuclei, locus coeruleus and small cells within the mesencephalic nucleus, there were some Fos-IR neurons but a more variable incidence that did not reach significance. The bilateral transient response of the SolM neurons was so

consistent that we used that site for standardizing each set of immunoreactions (see Section 2).

3.4. Delayed transient Fos responses

At 1–2 weeks after PX and PXF injury, but not at 4 weeks or in the acute PX group, we found Fos-IR neurons in large ipsilateral neurons of the dorsomedial portion of nucleus oralis, including some large neurons of the adjacent reticular nucleus-_A (terminology of Ref. [60]) in a region that receives primary trigeminal afferent input (Figs. 4 and 5). There were also Fos-IR paratrigeminal neurons near the periobex trigeminal tract with a delayed, transient response at 1–2 weeks (Fig. 5). The Fos-IR neurons of the trigeminal tract had a significant ipsilateral response at 1

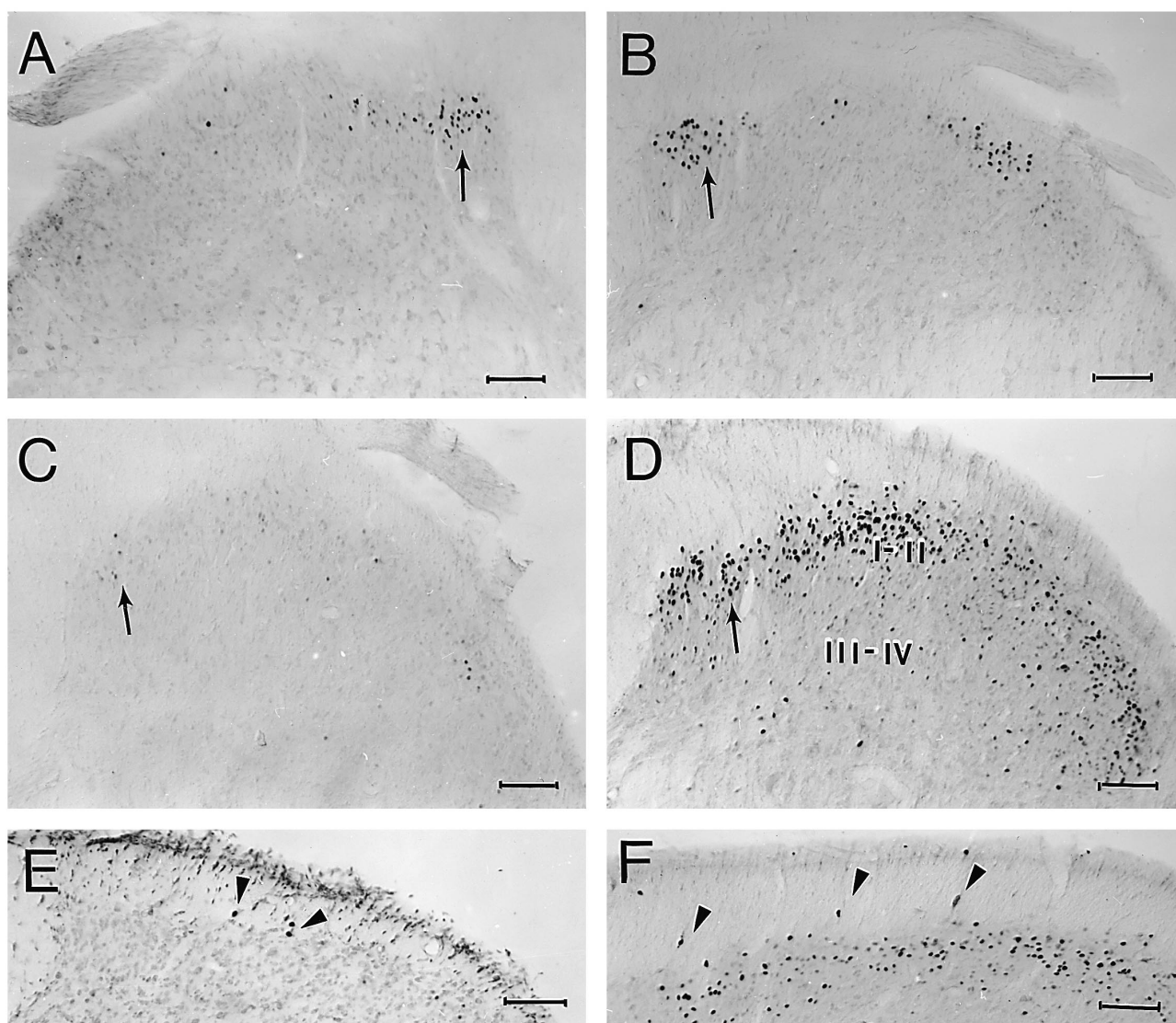


Fig. 8. Persistent Fos in cervical and medullary dorsal horn. (A,B) After 4 weeks PX there was a bilateral response in medial superficial neurons (arrow) of cervical contralateral (A) and ipsilateral (B) dorsal horn, compared to intact controls (C) and to the intense response after 2 weeks PXF (D). The interstitial cells of trigeminal tract of medullary dorsal horn had a persistent response in the 1–2-week PX groups (E) that was intensified for 2-week PXF (F). Abbreviations: I–II, superficial laminae; III–IV, deep laminae. Scales: 0.1 mm.

week for the PX group and much stronger bilateral response at 2 weeks for the PXF group (Fig. 5).

3.5. Persisting and late Fos responses

There were two different temporal patterns among the neurons with Fos-IR at 4 weeks after PX injury (Figs. 6–9): (1) an *ipsilateral* response that was significant at 1 week and then persisted at least 4 weeks (rostral lateral solitary nucleus, large neurons of dorsal nucleus caudalis) with a similar but not significant trend in ventral nucleus caudalis, and (2) a delayed response in neurons at 2–4 weeks (bilateral cervical dorsal horn, contralateral rostro-lateral solitary nucleus, contralateral nucleus caudalis). For each area the PXF group had many more reactive neurons than the PX groups. In some cases, the reactive neurons were predominantly ipsilateral (dorsal nucleus caudalis) and in others were bilateral (ventral nucleus caudalis, medial cervical dorsal horn). The Fos-IR neurons were located in laminae I and II for the PX injuries, whereas the filled PXF group also included neurons in deeper laminae (Fig. 8).

3.6. Cytochemistry of Fos-reactive neurons and adjacent afferent fibers

Most of the Fos-IR neurons were found in regions of brainstem where central Dyn-IR terminals colocalized with the Fos-IR neurons. These neurons were especially evident in interstitial regions of trigeminal tract, rostral lateral solitary nucleus, lamina I and II of nucleus caudalis, and medullary and cervical dorsal horn. A few neurons contained both Fos and Dyn under the conditions of these experiments (no colchicine treatment to enhance perikaryal peptides), and they were found in the ipsilateral SolL dorsal nucleus caudalis and trigeminal tract (Fig. 10).

Primary afferent arborization and IR for CGRP and p75NTR were each examined in adjacent sections to those reacted for Fos for all brainstem regions of each post-operative group using blind analyses of coded slides (NIH Image 1.62 density analysis of a standard area [0.25 mm²] at a standard threshold). The patterns within each animal were quite variable, and when three successive 50- μ m semi-sections per rat per nucleus were analyzed, no significant differences were found (data not shown).

3.7. Sizes of Fos-reactive nuclei

Measurements of the cross-sectional area of the Fos-IR nuclei in digital images at 1250 \times (Fig. 11) demonstrated that nuclei were significantly larger for ipsilateral neurons in the nucleus oralis/reticular zone, the trigeminal tract, and dorsal nucleus caudalis ($p < 0.05$) for the 1–4-week PX and 2-week PXF rats, but not for those of intact or DF groups, and not for rostral lateral solitary nucleus or contralateral Fos-IR neurons. Most of the Dyn/Fos double-labeled cells (Fig. 10) had large nuclei. The large nuclei were rare in ventral and overlap regions of trigeminal nucleus caudalis (data not shown). The similar high magnification figures (Figs. 2C–E, 4B, 6C,E, 9B,D–E) allow comparison of nuclear size for different areas and post-operative times.

3.8. Summary of Fos patterns after PX injury

The locations of Fos-IR neurons at 1–4 weeks after PX injury are summarized in Table 2. There were three different temporal patterns: (1) persistent Fos-IR neurons in the 1-, 2-, and 4-week groups, (2) delayed transient neurons that were only present at 1–2 weeks, and (3) a late group at 4 weeks. None of these was present in the DF groups, and all were present at more intense levels in the PXF group at 2 weeks, as shown in Figs. 5, 7 and 9.

4. Discussion

In the present study, the expression of Fos-IR in brainstem and cervical neurons were analyzed after three types of dental injury in adult rat molars and compared with Fos patterns in intact control rats. The study was designed to analyze the PX group at post-operative times from an acute period (5–7 h) to 1, 2, and 4 weeks. Previous studies demonstrated persisting cytochemical effects in the primary afferent neurons with PX injury [68,77] compared to lack of such changes after mild DF injury to the molar teeth. The latter procedure causes transient nerve sprouting beneath the cavity site and nearby pulpal reactions that heal within 1–2 weeks [73]. The pilot studies demonstrated lack of persistent Fos for rats with DF injury [15], so that we were able to have the DF group also serve as surgical sham controls for the PX group.

The PX injury initially destroys superficial coronal pulp and then gradually causes progressive pupal loss and irreversible pulpitis during subsequent weeks, with abscesses

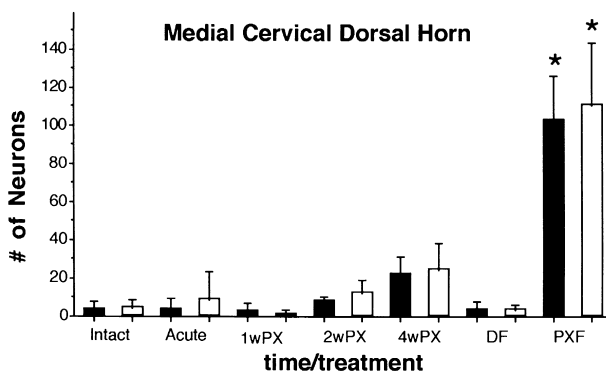
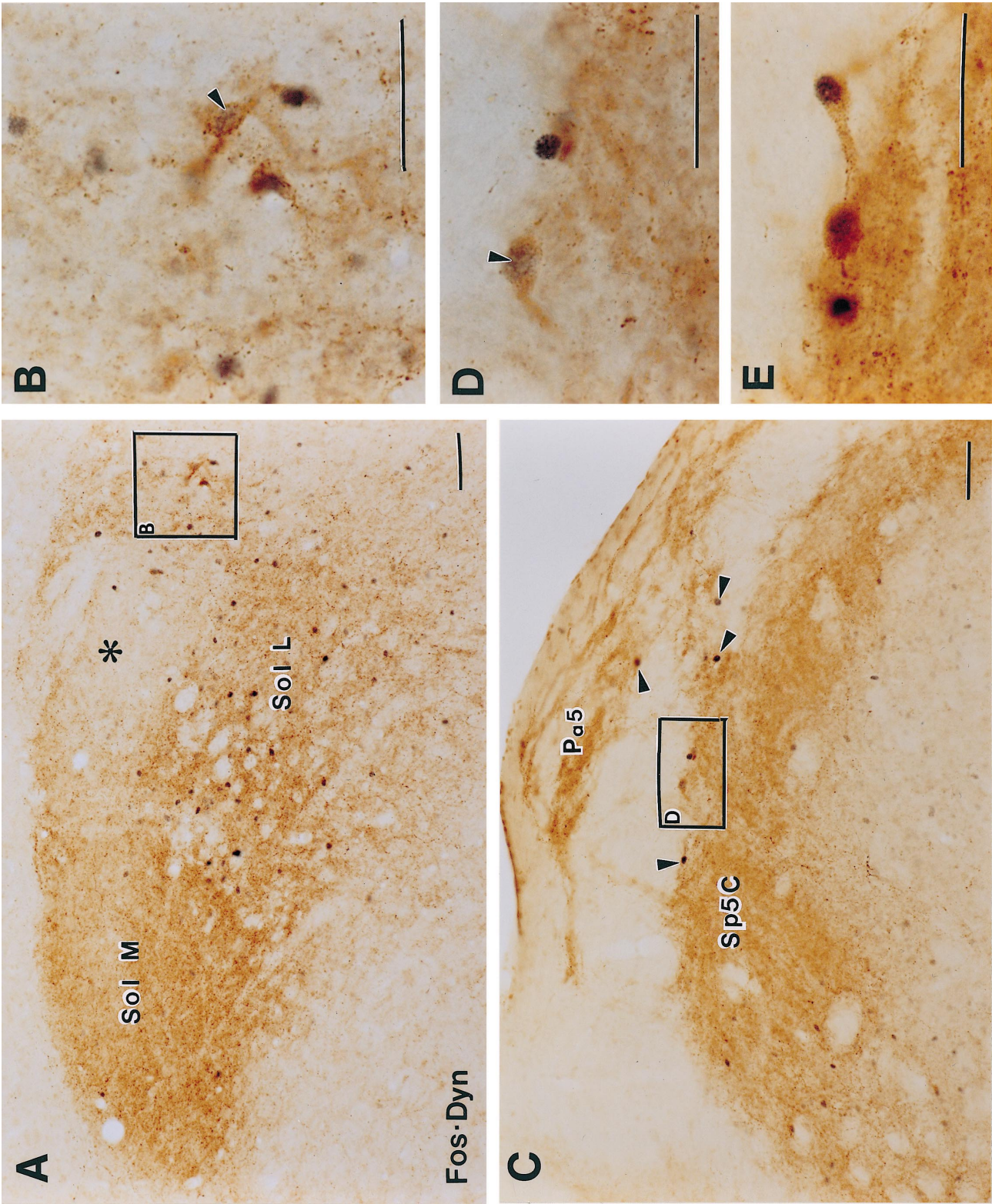


Fig. 9. Medial side of superficial dorsal horn in cervical C1–2. Data for the medial cervical dorsal horn were averaged from five successive sections per rat and only the PXF response was significant, although there was a strong bilateral trend for the 4-week PX group.



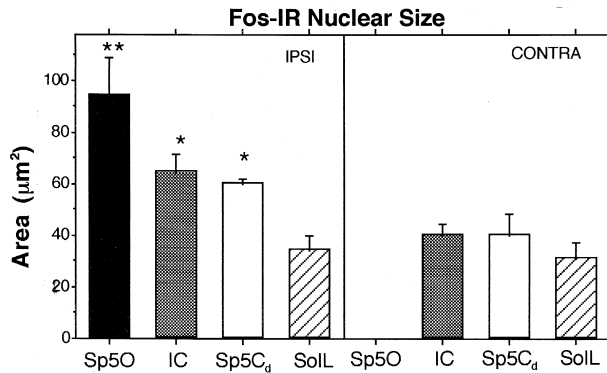


Fig. 11. Nuclear size in Fos-IR neurons. Comparison of the maximal cross-sectional area of the largest 10 Fos-IR nuclei contained within each section per region for the 1-week PX group. There was a significant difference between ipsilateral oralis neurons (Sp5O, black bar) and others on the ipsilateral side (** $p < 0.05$) and between the ipsilateral and contralateral dorsal nucleus caudalis (Sp5Cd, white bars, NCD) and interstitial cells of trigeminal tract (IC, gray bars) (mean \pm S.D.) (* $p < 0.02$). SolL (striped bars) nuclear sizes were similar for both sides.

extending out into periodontal ligament and alveolar bone by 3–5 weeks [45,46]. Thus, there is a persistent acute inflammation zone at the interface of abscessed and vital tissue that shifts location from inside the tooth for the 1-week group to progressively involved periodontal tissues for the 2- and 4-week groups (Fig. 1). The shifting Fos patterns for the 1-, 2-, and 4-week groups are discussed below in relation to the peripheral events.

Despite the significant peripheral pathology for PX lesions, the rats resumed their weight gain by 2–5 days and did not display subsequent aversive feeding or other abnormal behaviors. We, therefore, added a small group of rats with PXF lesions, for which the return to post-operative weight gain was more prolonged (5–7 days) in order to see whether there were greater Fos-IR neuron numbers and distribution at 2 weeks after injury for conditions that cause greater aversive feeding behavior. This hypothesis was supported by the data from the PXF group, and is being further analyzed in on-going studies.

Increased synthesis and nuclear location of the transcription factor c-Fos has been used during the past decade as a precise indicator of acute and persistent central neuronal reactions to peripheral injury, inflammation and neuropathy [1,9,26,28,32,33,37,41,49,52,55,57,58,61,70]. In previous studies of acute Fos-IR responses after dental injury, neurons in medullary and cervical dorsal horn expressed Fos regardless of whether the stimulus was by pulpal mechanical damage, noxious heat, electrical stimuli, tooth movement or tooth extraction, and with similar re-

Table 2

Fos-IR expression patterns after pulp exposure injury

	Ipsilateral	Contralateral
Delayed transient ^a	dorsal Sp50 paratrigeminal nucleus sp5 — interstitial cells	
Persistent	rostral SolL dorsal peribex Sp5C	
Late response	laminae I–II, C1–2	rostral SolL dorsal peribex Sp5C laminae I–II, C1–2

^aDelayed transient pattern was found at 1–2 weeks; persistent pattern was still strong at 4 weeks; and late response was significant by 2–4 weeks.

sults for rat, cat and ferret [3,23,42,44,59,76]. In all cases, there were Fos-IR neurons in superficial laminae of dorsal nucleus caudalis, especially at the interpolaris/caudalis transition zone, as we reported initially [15]. Electrical stimulation of A- β nerve fibers at the threshold intensities to elicit the jaw opening reflex in cat canine teeth did not evoke a Fos response, but as the stimulus intensity was increased 2–6 times over threshold there was a progressive recruitment of A- δ and C-fibers, increased numbers of Fos-IR neurons, increased rostro-caudal extent of Fos-IR, and the addition of Fos-IR neurons in deeper laminae [42]. Dentinal hydrodynamic (osmotic) stimulation (A-fiber specific) of ferret teeth elicited Fos-IR neurons in ipsilateral dorsal nucleus caudalis only, whereas noxious heat stimuli caused bilateral Fos-IR in ventral regions of the interpolaris/caudalis transitions zone [19].

The present study was similar to previous analyses of dental injury and central Fos in having an acute bilateral response in SolM and LRT, and in having a different rostro-caudal extent and intensity of the response with different durations or severity of injury. It differs from the previous ones in several ways. First, we did not find a significant Fos response in nucleus caudalis in the acute (5–7 h) group after rat molar pulp exposure. The PX injury appears to be below threshold for causing significant acute Fos responses while PXF gave a much stronger response at the time examined (2 weeks). Similarly, electrical stimuli are not sufficient to induce a Fos response until the intensity is more than double that required for jaw opening reflex threshold [42,59]. At 5–7 h after injury, the peripheral endings of sensory fibers were still depleted of CGRP near the damaged pulp (Fig. 1).

Second, we have shown that the PX or PXF injury caused a delayed, transient expression of Fos at 1–2 weeks

Fig. 10. Dyn/Fos double-labeling (A,B). At 1 week after a PX injury, double-labeled cells for Fos (gray/black) and Dyn (orange) were found on the lateral side of the rostral–lateral solitary nucleus, shown at higher magnification in (B). Many of the interstitial and marginal cells (arrowheads) in or next to the trigeminal tract at peribex nucleus caudalis (C,D), and in the medullary dorsal horn (E) were double-labeled for Fos and Dyn. The small dark nucleus in (D) is superimposed on a double-labeled cell located deeper in the section. Even though the responses in peribex nucleus caudalis (Sp5C) and paratrigeminal nucleus (Pa5) were weak in this rat, there was a strong response of the interstitial neurons. Scale bars: 0.1 mm.

in large oralis/reticular neurons. By contrast, electrophysiological studies have shown that the receptive field plasticity of oralis neurons after molar pulp exposure injury in rats was not significant until 4 weeks after injury [74]. Fos-IR has also been reported in nucleus oralis after electrical tooth stimulation of ferret canines [59], in which the electrodes were implanted 8 days prior to stimulation to allow recovery from the implantation procedure, and in rats after mechanical injury of incisor pulp [72].

Third, we found unusual reactivity in (1) the neurons that occur within the trigeminal tract in the paratrigeminal nucleus (also called interstitial nucleus [17]), (2) the dispersed interstitial cell islands within the tract, and (3) the marginal zone next to lamina I of the medullary dorsal horn and nucleus caudalis. Those neurons were found from the edges of the paratrigeminal nucleus in the periobex region, along the marginal edge of the dorsal nucleus caudalis and extending along the tract throughout the medulla; and all were located among Dyn-IR fibers that pass between the main fibers of the tract (Fig. 10). They differed from the neurons in the main part of the paratrigeminal nucleus which only rarely had a Fos response. Interstitial and marginal cells of the trigeminal tract display a strong Fos response to noxious chemical stimulation of temporomandibular joint [34], but not to chemical stimulation of tongue [17], whereas the latter study found intense responses within the paratrigeminal nucleus and the former had only a few Fos-IR neurons in paratrigeminal nucleus. Clearly, the small clusters of neurons within the trigeminal tract have subtle differences in their Fos responses to peripheral inflammation. In the present study, only the marginal and interstitial cells near the dorsal nucleus caudalis were activated by PX lesions, while the PXF group also included ventral tract neurons.

Previous anatomical and physiological studies of dental afferents found extensive ipsilateral connections at all levels of the trigeminal brainstem subnuclei, as demonstrated by retrograde-labeling of primary afferents from teeth [51], by [^{14}C]2-deoxyglucose-labeling after tooth stimulation [67], and by numerous electrophysiological studies [25,48,66,74]. One might therefore have expected a wider distribution in brainstem and cervical cord of Fos-IR neurons after pulp exposure injury, rather than the focal concentrations reported here. This situation is similar to that for noxious stimulation of cornea [70], dura vessels [69] and temporomandibular joint [34], each of which has a wide distribution of electrophysiologically responsive neurons from cervical cord throughout the trigeminal subnuclei but Fos-IR neurons clustered in the periobex region and cervical dorsal horn [5]. Most dental stimuli, including those used in some Fos studies [23], cause pain sensations [7], and many physiological works indicate that trigeminal pain stimuli enter the CNS primarily through the nucleus caudalis and C1 dorsal horn [66]. The lack of Fos-IR response in the other neurons receiving the rest of dental (or corneal, dural or joint) afferent connections in the

brainstem shows that long-term plasticity in those central neurons does not require Fos mechanisms.

The interpolaris/caudalis transition zone and associated interstitial tract neurons have been proposed to be a special trigeminal subnucleus [3,5,34,70] with autonomic and nociceptive input. Our study proposes a further subdivision within that region, because the ipsilateral interstitial and marginal neurons of that region and some superficial neurons of nucleus caudalis had large nuclei and were Dyn-rich during persistent tooth PX injury (Fig. 10), whereas the rest of ipsilateral nucleus caudalis neurons and all contralateral Fos-IR neurons had smaller nuclei and did not have detectable co-expression of Dyn.

In the present study, the pulp exposure injuries (PX and PXF) induced a progressive acute inflammation (vasodilation, polymorphonuclear leukocyte extravasation, sensory nerve sprouting) that persisted along the border of surviving vital pulp next to an expanding necrotic zone. The necrosis gradually consumed the pulp and extended into the periodontal ligament after several weeks (Fig. 1), as reported previously [46]. Some Fos patterns may depend on particular phases of that injury process. For example, transient Fos at nucleus oralis at 1 week may be driven by conditions that only last while some crown pulp remains vital. By 4 weeks, most of the pulp had disappeared and the afferent nerve fibers of periodontal ligament were affected by inflammation and remodeling. Those peripheral conditions would elicit abnormal activity in differing sets of afferents from the earlier period when the lesion was located in the crown.

Serial sections from C1 to the level of the caudal mesencephalic nucleus were examined using antisera to CGRP and p75NTR in all the groups of rats representing all post-operative times. We had expected to find alterations in primary afferent distribution or cytochemistry in the dorsal horn of brainstem, or at least in those regions with a Fos response, based on the upregulation of p75-NTR in the trigeminal ganglion after rat molar injury [78] and the increased CGRP-IR and p75NTR-IR of peripheral dental fibers that sprout near injuries [16,46]. However, no significant differences were found for the distribution or cytochemistry of trigeminal afferent endings for any of the injury paradigms or post-operative durations. Previous demonstrations of altered central endings after trigeminal injury involved deafferentation of vibrissae and only found subtle changes if the animals were immature [63]. Dental and vibrissal afferents therefore appear to have different central responses from those causing A β sprouting in spinal dorsal horn after spinal nerve injury [80].

A correlation of *c-fos* induction and Dyn expression has been shown for spinal cord [26,39,40,53,54,64] with over 80% of the Fos-IR neurons co-expressing Dyn [58]. Detection of peptide was enhanced in the latter study by colchicine treatment to block transport of peptide out of the cell body. In the present study, we did not utilize colchicine treatment (to avoid manipulation of the animals

during the last day), but were able to demonstrate colocalization of Dyn and Fos in the large neurons of the ipsilateral dorsal nucleus caudalis, interstitial cells of the trigeminal tract, and large neurons along the lateral edge of SolL. Double-labeled neurons were not found in intact rats or in injured rats after shallow drill/fill injuries, contralaterally after pulp exposure injury, or in other ipsilateral sites such as ventral nucleus caudalis.

We have also found significantly larger Fos-IR nuclei in some ipsilateral sites, such as dorsal nucleus caudalis, nucleus oralis, and the Dyn-rich interstitial cells of the trigeminal tract. The lack of large nuclei contralaterally suggests that different central neurons are participating in the Fos response on different sides of the brainstem, despite their similar laminar distribution. Another possibility is that some nuclei may enlarge during the prolonged nociceptive stimulation that is induced by pulp lesions. Nuclear enlargement has been reported for some cellular responses to tissue damage, for example in injured/regenerating myocytes that exhibit increased cell size [30] or for glia at epileptic foci [47]. Further work is needed to determine the significance of the large nuclei in ipsilateral Fos-IR neurons responding to persistent dental inflammation, and to further investigate the subtle differences in the central responses that occur at different times and for different types of dental injury.

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